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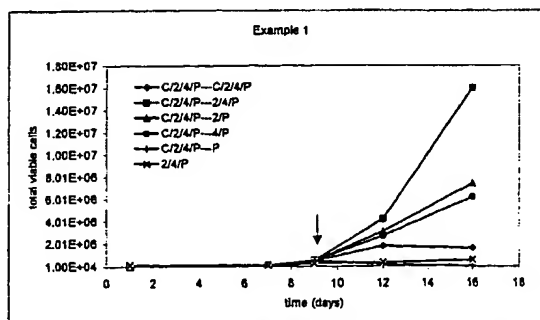
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ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.

(54) Title: PRODUCTION OF TcR GAMMA DELTA T CELLS



EXPT. Condition	1 day	7 days	9 days	12 days	16 days	Percent TcRγδ+
C/2/4/P-C/2/4/P	2.60E+04	1.10E+05	5.00E+05	1.80E+06	1.60E+07	97
C/2/4/P-2/4/P	2.60E+04	1.10E+05	5.00E+05	2.30E+06	1.60E+07	99
C/2/4/P-2/P	2.60E+04	1.10E+05	5.00E+05	3.10E+06	7.40E+06	99
C/2/4/P-4/P	2.60E+04	1.10E+05	5.00E+05	2.70E+06	6.20E+06	98
C/2/4/P-P	2.60E+04	1.10E+05	5.00E+05	1.60E+05	1.90E+04	nd
2/4/P	1.30E+03	1.10E+05	2.50E+05	3.40E+05	5.30E+05	56

C = Concanavalin A
2 = Interleukin-2
4 = Interleukin-4
P = Autologous plasma
— = sub-culture
nd = not done

(57) Abstract: The method for obtaining and expanding TcRγδ⁺ T cells in culture is described. The method involves: (1) culturing cells from a sample containing TcRγδ⁺ T cells or precursors thereof in a first culture medium comprising a T cell mitogen and at least two growth factors and (2) culturing the cells obtained in step (1) in a second culture medium comprising at least two growth factors. The two growth factors are factors with interleukin-2-like and interleukin-7-like activity. The TcRγδ⁺ T cells obtained by the method can be used in a variety of experimental, therapeutic and commercial applications.

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Title: Production of TcR Gamma Delta T Cells

FIELD OF THE INVENTION

The present invention relates to novel and improved methods for the *ex vivo* expansion of TcR $\gamma\delta^+$ T cells.

5 **BACKGROUND OF THE INVENTION**

TcR $\gamma\delta^+$ T cells are a small subset of circulating T lymphocytes that are distinct from conventional TcR $\alpha\beta^+$ T cells which recognize, with fine specificity, foreign peptide antigens in the context of classical class I or class II major histocompatibility complex (MHC) restriction elements. By contrast,
10 TcR $\gamma\delta^+$ T cells are able to recognize both peptide and non-peptide antigens which may be derived from either foreign microorganisms or endogenous cellular products induced by stress such as viral infection or transformation. Moreover, unlike antigen recognition by TcR $\alpha\beta^+$ T cells, antigen recognition by TcR $\gamma\delta^+$ T cells is not MHC-restricted.

15 The T cell receptors of TcR $\alpha\beta^+$ and TcR $\gamma\delta^+$ T cells are distinguished by the different genetic elements that encode them. The majority of TcR $\gamma\delta^+$ T cells are classified into two main subsets, V $\delta 1^+$ and V $\delta 2^+$, based on the genes that encode their δ chain. The major subset of TcR $\gamma\delta^+$ T cells in human peripheral blood expresses V $\delta 2$ in combination with V $\gamma 9$, while most of the
20 remainder express V $\delta 1$ in combination with V $\gamma 2$, V $\gamma 3$, V $\gamma 4$, V $\gamma 5$ or V $\gamma 8$ (Salerno, A. and Dieli, F., 1998).

Since TcR $\gamma\delta^+$ T cells lack the fine specificity characteristics of TcR $\alpha\beta^+$ T cells, it has been proposed that they represent a more primitive immune mechanism that provides a first-line surveillance function against infection
25 and tumours (Boismenu, R. et al., 1997). Several studies have documented the response of TcR $\gamma\delta^+$ T cells to various viruses, bacteria and parasites (Bukowski, J.F. et al., 1994; Wallace, M. et al., 1995; Lang, F. et al., 1995; Elloso, M.M. et al., 1996) as well as their ability to mediate lysis of tumour cells of various origins (Zocchi, M.R. et al., 1990; Kitayama, J. et al., 1993; Choudhary,
30 A. et al., 1995). Hematopoietic tumours may be particularly susceptible to the lytic effects of TcR $\gamma\delta^+$ T cells, since transgenic mice expressing the V $\gamma 1.1$ transgene display spontaneous resistance to injected T cell leukemias, and

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TcR $\gamma\delta^+$ T cell hybridomas derived from these mice preferentially respond to hematopoietic malignant cells over non-hematopoietic tumour cells (Penninger, J. et al., 1995). Moreover, human TcR $\gamma\delta^+$ T cell clones derived from patient peripheral blood and bone marrow have been shown to lyse
5 autologous leukemic cells in acute lymphoblastic leukemia and acute myeloid leukemia, respectively (Bensussan, A. et al., 1989; Jahn, B. et al., 1995). Furthermore, improved disease-free survival in leukemia patients after allogeneic bone marrow transplantation has been shown to be associated with an increase in the number and percentage of TcR $\gamma\delta^+$ T cells in peripheral
10 blood (Lamb, L.S. et al., 1996). Collectively, these results suggest that TcR $\gamma\delta^+$ T cells may have therapeutic potential in the treatment of cancer and infectious diseases.

Many of the published methods describing the *ex vivo* expansion of TcR $\gamma\delta^+$ T cells require the presence of antigen. Virus-infected or transformed
15 cells or cell lines, bacteria and parasites have been shown to stimulate TcR $\gamma\delta^+$ T cell expansion *ex vivo*, as have established tumour cell lines. For example, herpes simplex virus (HSV)-infected cells were used to stimulate the expansion of V $\delta 2^+$ cells (Bukowski, J.F. et al., 1994), while Epstein-Barr virus (EBV)-transformed B-lymphoblastoid cell lines were used to stimulate the
20 expansion of V $\delta 1^+$ cells (Orsini, D.L.M. et al., 1993). Extracts of *Mycobacterium tuberculosis* and blood-stage *Plasmodium falciparum* malarial antigens have been shown to stimulate proliferation of TcR $\gamma\delta^+$ T cells (Constant, P. et al., 1994; Elloso, M.M. et al., 1996). Daudi, an immortalized human Burkitt's lymphoma cell line, can also stimulate the proliferation of TcR $\gamma\delta^+$ T cells (Kaur,
25 I. et al., 1993). In addition, well-characterized, non-peptidyl antigens of the prenyl phosphate family, for example, isopentenyl pyrophosphate, have been shown to stimulate the *ex vivo* expansion of TcR $\gamma\delta^+$ T cells (Garcia, V.E. et al., 1998). In some of these systems, the antigen-stimulated cultures of TcR $\gamma\delta^+$ T cells were supplemented with cytokines.

30 TcR $\gamma\delta^+$ T cells have also been expanded *ex vivo* from populations of tumour infiltrating lymphocytes (TIL) by culture with IL-2 (Zocchi, M.R. et al., 1990) or IL-2 in combination with immobilized anti-CD3 antibody (Kitayama,

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J. et al., 1993) or anti-TcR $\gamma\delta$ antibody (Yu, S. et al., 1999). In these systems, selective stimulation of the TcR $\gamma\delta^+$ T cells by the tumour antigens is presumed to have occurred *in vivo* prior to isolation of T cells from the cancerous tissue.

In another system, TcR $\gamma\delta^+$ T cells were expanded from the peripheral
5 blood of glioblastoma patients using a solid-phase, immobilized anti-CD3 antibody in combination with IL-2 followed by culture in IL-2 alone (Yamaguchi, T., et al, 1997). These authors reported that the subsequently purified TcR $\gamma\delta^+$ T cells did not proliferate for more than one week in the presence of IL-2 alone and therefore, they concluded, that this method would
10 be applicable only to short term studies. They further showed that the method resulted in the expansion and enrichment of both TcR $\gamma\delta^+$ and TcR $\alpha\beta^+$ T cells, achieving TcR $\gamma\delta^+$ T cell purities on the order of 28%. In a subsequent report, the same authors demonstrated that this method selectively expanded the V $\delta 2^+$ subset (Suzuki, Y., et al, 1999). In another report, this group showed
15 that TcR $\gamma\delta^+$ T cells which were expanded in culture using anti-CD3 and IL-2 followed by IL-2 alone and which were subsequently purified from the expanded population proliferated better in a ^3H -thymidine incorporation assay when IL-15 was added (Yamaguchi, et al, 1998).

Thus, there are limitations to cell proliferation and/or requirements for
20 antigen stimulation in the existing methods for *ex vivo* culture and expansion of TcR $\gamma\delta^+$ T cells. In view of the foregoing, there is a need in the art for a method to selectively culture large amounts of essentially pure TcR $\gamma\delta^+$ cells *in vitro*.

SUMMARY OF THE INVENTION

25 The present invention provides novel methods for expanding TcR $\gamma\delta^+$ T cells in culture in the absence of exogenous antigen. In particular, the inventors have shown that TcR $\gamma\delta^+$ T cells can be expanded by culturing the cells in a first media containing a T cell mitogen, interleukin 2 and interleukin 7 and then sub-culturing the cells in a second culture medium containing
30 interleukin 2 and interleukin 7 in the absence of the mitogen. Accordingly, the present invention provides a method for expanding TcR $\gamma\delta^+$ T cells in a starting sample comprising:

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- (1) culturing cells in the starting sample in a first culture medium comprising (a) a T cell mitogen, (b) a growth factor having interleukin-2-like activity and (c) a growth factor having interleukin-7-like activity; and
- 5 (2) culturing the cells obtained in step (1) in a second culture medium comprising (i) a growth factor having interleukin-2-like activity and (ii) a growth factor having interleukin-7-like activity to expand TcR $\gamma\delta^+$ T cells.

The term "a growth factor having interleukin-2-like activity" means
10 any compound that has the same activity as IL-2 with respect to its ability to expand TcR $\gamma\delta^+$ T cells in culture and includes, but is not limited to, IL-2 and IL-2 mimetics, or any functional equivalent of IL-2.

The term "a growth factor having interleukin-7-like activity" means
any compound that has the same activity as IL-7 with respect to its ability to
15 expand TcR $\gamma\delta^+$ T cells in culture and includes, but is not limited to, IL-7, IL-7 mimetics, or any functional equivalent of IL-7.

The methods of the invention result in expanded cell populations of TcR $\gamma\delta^+$ T cells. By "expanded" it is meant that the number of the desired or target cell type (i.e., TcR $\gamma\delta^+$ T cells) in the final preparation is higher than the
20 number in the initial or starting cell population.

In a preferred embodiment, prior to culturing the cells in the first culture medium, the cells are depleted of non-TcR $\gamma\delta^+$ cells. The first and second culture media may also contain other growth factors (in addition to IL-2 and IL-7) that can enhance the expansion of the TcR $\gamma\delta^+$ T cells. Examples
25 of such growth factors include IL-4 and IL-15.

The TcR $\gamma\delta^+$ T cells obtained by the method of the invention can be used in a variety of experimental, therapeutic and commercial applications.

Other features and advantages of the present invention will become apparent from the following detailed description. It should be understood,
30 however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and

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scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention will now be described in relation to the drawings in
5 which:

Figure 1 is a graph showing the total viable cells versus time under various culture conditions.

Figure 2 is a graph showing the total viable cells versus time under various culture conditions.

10 Figure 3 is a graph showing the total viable cells versus time under various culture conditions.

Figure 4 is a graph showing the total viable cells versus time under various culture conditions.

15 Figure 5 is a graph showing the total viable cells versus time under various culture conditions.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides novel methods for selectively expanding TcR $\gamma\delta^+$ T cells in culture. The methods can use either unfractionated starting samples or starting samples which have been enriched
20 for T cells. Advantageously, the methods of the invention do not require the use of antigenic stimulation which is necessary in most other procedures.

Accordingly, the present invention provides a method for expanding TcR $\gamma\delta^+$ T cells in a starting sample comprising:

- 25 (1) culturing cells in the starting sample in a first culture medium comprising a T cell mitogen and at least two growth factors; and
- (2) culturing the cells obtained in step (1) in a second culture medium comprising at least two growth factors to expand TcR $\gamma\delta^+$ T cells.

The two growth factors can be any growth factors that can expand TcR $\gamma\delta^+$ T cells in culture such as IL-2 and IL-7 or growth factors that have the
30 same activity as IL-2 or IL-7 and can expand TcR $\gamma\delta^+$ T cells in culture.

In one aspect, the present invention provides a method for expanding TcR $\gamma\delta^+$ T cells in a starting sample comprising:

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- (1) culturing cells in the starting sample in a first culture medium comprising (a) a T cell mitogen, (b) a growth factor having interleukin-2-like activity and (c) a growth factor having interleukin-7-like activity; and
- 5 (2) culturing the cells obtained in step (1) in a second culture medium comprising (i) a growth factor having interleukin-2-like activity and (ii) a growth factor having interleukin-7-like activity to expand TcR $\gamma\delta^+$ T cells.

The term "a growth factor having interleukin-2-like activity" means
10 any compound that has the same activity as IL-2 with respect to its ability to expand TcR $\gamma\delta^+$ T cells in culture and includes, but is not limited to, IL-2 and IL-2 mimetics, or any functional equivalent of IL-2.

The term "a growth factor having interleukin-7-like activity" means
any compound that has the same activity as IL-7 with respect to its ability to
15 expand TcR $\gamma\delta^+$ T cells in culture and includes, but is not limited to, IL-7, IL-7 mimetics, or any functional equivalent of IL-7.

The starting sample can be any sample that contains TcR $\gamma\delta^+$ T cells or precursors thereof including, but not limited to, blood, bone marrow, lymphoid tissue, epithelia, thymus, liver, spleen, cancerous tissues, lymph
20 node tissue, infected tissue, fetal tissue and fractions or enriched portions thereof. The starting sample is preferably blood including peripheral blood or umbilical cord blood or fractions thereof, including buffy coat cells, mononuclear cells and low density mononuclear cells (LDMNC). The cells may be obtained from a starting sample of blood using techniques known in
25 the art such as density gradient centrifugation. For example, whole blood may be layered onto an equal volume of Ficoll-Hypaque™ followed by centrifugation at 400xg for 30 minutes at room temperature. The interface material will contain the low density mononuclear cells which can be collected and washed in culture medium and centrifuged at 100xg for 10 minutes at
30 room temperature. Prior to culturing for TcR $\gamma\delta^+$ cells, the cells can be maintained in any suitable mammalian culture medium such as AIM-V™, RPMI 1640 or IMDM.

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Prior to culturing the starting sample or fraction thereof (such as LDMNC) in the first culture medium, the sample or fraction thereof may be enriched for certain cell types and/or depleted for other cell types. In particular, the starting sample or fraction thereof may be enriched for T cells together with the depletion of TcR $\alpha\beta$ ⁺ T cells. The sample may be enriched or
5 depleted of certain cell types using techniques known in the art. In one embodiment, the cells of a particular phenotype may be depleted by culturing the starting sample or fraction thereof with an antibody cocktail containing antibodies specific for markers on the cells to be depleted. Preferably, the
10 antibodies in the cocktail are tetrameric antibody complexes as described in United States Patent No. 4,868,109 to Lansdorp.

Once the cells in the starting sample have been fractionated and enriched, if desired, the cells are cultured in a first culture medium comprising a T cell mitogen and at least two growth factors, preferably a growth factor
15 having interleukin-2-like activity and a growth factor having interleukin-7-like activity. Preferably, the T cell mitogen is present in an amount from about 0.01 to about 100 $\mu\text{g/ml}$; the growth factor having interleukin-2-like activity is present in an amount from about 0.1 to about 1000 ng/ml; the growth factor having interleukin-7-like activity is present in an amount from
20 about 0.1 to about 1000 ng/ml. More preferably, the T cell mitogen is present in an amount from about 0.1 to about 50 $\mu\text{g/ml}$; the growth factor having interleukin-2-like activity is present in an amount from about 1 to about 100 ng/ml; the growth factor having interleukin-7-like activity is present in an amount from about 1 to about 100 ng/ml. Most preferably, the medium
25 comprises 1 $\mu\text{g/mL}$ of a T cell mitogen; 10 ng/mL of a growth factor having interleukin-2-like activity and 10 ng/mL of a growth factor having interleukin-7-like activity.

The cells are preferably cultured in the first culture medium for a period of time ranging from about 3 days to about 21 days. More preferably,
30 from about 5 days to about 14 days.

The T cell mitogen can be any agent that can stimulate T cells including, but not limited to, lectins of plant and non-plant origin, antibodies that activate T cells, and other non-lectin/non-antibody mitogens. A preferred

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plant lectin is concanavalin A (ConA) although other plant lectins such as phytohemagglutinin (PHA) may be used. Preferred antibodies include an anti-CD3 antibody such as OKT3, an anti-CD2 antibody such as OKT11 or an anti-CD28 antibody. Within the context of the present invention, antibodies
5 are understood to include monoclonal antibodies, polyclonal antibodies, antibody fragments (e.g., Fab, and F(ab')₂), single chain antibodies, single chain variable fragments (ScFv) and recombinantly produced binding partners. Other mitogens include phorbol 12-myristate-13-acetate (TPA) and its related compounds, such as mezerein, or bacterial compounds such as
10 Staphylococcal enterotoxin A (SEA) and Streptococcal protein A. The T cell mitogen may be soluble or immobilized and more than one T cell mitogen may be used in the method of the invention.

Following culture in the first culture medium, the cells are washed by centrifugation and sub-cultured in a second culture medium comprising at
15 least two growth factors, preferably a growth factor having interleukin-2-like activity and a growth factor having interleukin-7-like activity. If the cells are sub-cultured with IL-2 alone then proliferation continues for a few days but then quickly abates. Thus, for continued cell proliferation following the removal of the mitogen, growth factors with both IL-2 and IL-7-like activity
20 are required in the second culture medium.

The sub-culture step (i.e. removal of the T cell mitogen) is important for the expansion of TcR $\gamma\delta^+$ T cells by the method of the present invention particularly if the starting sample or LDMNC are not fractionated prior to culture in the first culture medium. If the LDMNC are fractionated then the
25 subculture step may be optional. Conversely, if mitogen is left out of the first culture medium, little/no cell expansion occurs. The removal of mitogen by sub-culture has the further advantage of making the TcR $\gamma\delta^+$ T cells better suited for therapeutic use, as the administration of residual mitogen to a patient is not desirable. The removal of the T cell mitogen in the subculturing
30 step may not be required if the TcR $\gamma\delta^+$ T cells are for experimental, diagnostic or other non-therapeutic uses.

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Preferably, in the second culture medium, the two growth factors are present in the same concentrations as listed above for the first culture medium.

The cells are preferably cultured in the second culture medium for a
5 period of time ranging from about 3 days to about 21 days. More preferably, from about 9 days to about 13 days.

The first and second culture media may additionally include other ingredients that can assist in the growth and expansion of the TcR $\gamma\delta^+$ T cells. Examples of other ingredients that may be added, include, but are not limited
10 to, plasma or serum, additional growth factors including cytokines such as IL-4 and IL-15, tumour necrosis factors (TNFs) and interferons (IFNs), purified proteins such as albumin, a lipid source such as low density lipoprotein (LDL), vitamins, amino acids, steroids and any other supplements supporting or promoting growth and/or survival.

15 In a preferred embodiment, the first and second culture media additionally include other growth factors such as IL-4 or IL-15 or a mimetic or functional equivalent thereof. The inventors have found that the addition of a third growth factor to the first and second media enhances the expansion of the TcR $\gamma\delta^+$ T cells as compared to the expansion obtained using two growth
20 factors.

Accordingly, the present invention provides a method for expanding TcR $\gamma\delta^+$ T cells in a starting sample comprising:

- (1) culturing cells in the starting sample in a first culture medium comprising a T cell mitogen and at least three growth factors; and
- 25 (2) culturing the cells obtained in step (1) in a second culture medium comprising at least three growth factors to expand TcR $\gamma\delta^+$ T cells.

In a preferred embodiment, the three growth factors are selected from combinations of IL-2, IL-4, IL-7; and IL-15. Examples of combinations include IL-2, IL-4 and IL-15; IL-2, IL-4 and IL-7 and IL-2, IL-7 and IL-15. The third
30 growth factor can be present in an amount similar to the two growth factors as described above.

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The inventors have also found that addition of a fourth growth factor to the first and second media enhances the expansion of the TcR $\gamma\delta^+$ T cells as compared to the expansion obtained using two or three growth factors.

Accordingly, the present invention provides a method for expanding
5 TcR $\gamma\delta^+$ T cells in a starting sample comprising:

- (1) culturing cells in the starting sample in a first culture medium comprising a T cell mitogen and at least four growth factors; and
- (2) culturing the cells obtained in step (1) in a second culture medium comprising at least four growth factors to expand TcR $\gamma\delta^+$ T cells.

10 In preferred embodiments, the four growth factors are IL-2, IL-4, IL-7 and IL-15.

Preferably, both the first and second culture media are supplemented with serum or plasma (P). The amount of P in the first and second culture media is preferably from about 1% to about 25%. More preferably, the
15 amount of P in the first and second culture media is from about 2% to about 20%. Even more preferably, the amount of P in the first and second culture media is from about 2.5% to about 10%. Most preferably, the amount of P in the first and second culture media is 5%. The serum or plasma (P) can be obtained from any source including, but not limited to, human peripheral
20 blood, umbilical cord blood, or blood derived from another mammalian species. The plasma may be from a single donor or may be pooled from several donors. If autologous TcR $\gamma\delta^+$ T cells are to be used clinically, i.e. re-infused into the same patient from whom the original starting sample was obtained, then it is preferable to use autologous P as well (i.e. from the same
25 patient) to avoid the introduction of extraneous products (e.g. viruses) into that patient. If the TcR $\gamma\delta^+$ T cells are to be used allogeneically (i.e. infused into a person other than the one from whom the original starting sample was obtained) then it is preferable to use plasma from one or the other to minimize the introduction of extraneous products into the patient; at a
30 minimum the plasma should be human-derived to avoid the administration of animal products to the patient.

The methods of the invention result in expanded cell populations of TcR $\gamma\delta^+$ T cells. By "expanded" it is meant that the number of the desired or

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target cell type (i.e., TcR $\gamma\delta^+$ T cells) in the final preparation is higher than the number in the initial or starting cell population.

The TcR $\gamma\delta^+$ T cells obtained according to the methods of the invention can be separated from other cells that may be present in the final culture
5 using techniques known in the art including fluorescence activated cell sorting, immunomagnetic separation, affinity column chromatography, density gradient centrifugation and cellular panning.

The present invention includes the TcR $\gamma\delta^+$ T cells obtained by the methods of the invention. Accordingly, the present invention provides a cell
10 preparation of TcR $\gamma\delta^+$ T cells. Preferably, the TcR $\gamma\delta^+$ T cells comprise greater than 60%, more preferably greater than 80% and most preferably greater than 90%, of the total cells in the enriched population.

The present invention also includes the use of the TcR $\gamma\delta^+$ T cells obtained by the method of the invention in any and all applications. TcR $\gamma\delta^+$ T
15 cells are thought to be a first line of defense against infectious pathogens. In addition, TcR $\gamma\delta^+$ T cells possess intrinsic cytolytic activity against transformed cells of various origins including B-cell lymphomas, sarcomas and carcinomas. As a result, the TcR $\gamma\delta^+$ T cells obtained and cultured *ex vivo* according to the method of the invention, can be transfused into a patient for the treatment or
20 prevention of infections, cancer or diseases resulting from immunosuppression. Advantageously, the TcR $\gamma\delta^+$ T cells of the invention do not contain mitogen or fetal bovine serum making them useful for human therapeutic applications. Accordingly, the present invention provides a method of modulating an immune response comprising administering an
25 effective amount of TcR $\gamma\delta^+$ T cells prepared according to a method of the invention to an animal in need thereof.

The term "effective amount" as used herein means an amount effective, at dosages and for periods of time necessary to achieve the desired results.

30 The term "animal" as used herein includes all members of the animal kingdom. Preferably, the animal is a mammal, more preferably a human.

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In one embodiment, the present invention provides a method of treating an infection comprising administering an effective amount of TcR $\gamma\delta^+$ T cells prepared according to the method of the invention to an animal in need thereof.

5 Examples of infections that may be treated include, but are not limited to, bacterial infections such as those caused by *Mycobacteria* (e.g. tuberculosis), viral infections such as those caused by herpes simplex virus (HSV), human immunodeficiency virus (HIV) or the hepatitis viruses, and parasitic infections such as those caused by *Plasmodium* (e.g. malaria).

10 In another embodiment, the present invention provides a method for treating cancer comprising administering an effective amount of TcR $\gamma\delta^+$ T cells prepared according to the method of the invention to an animal in need thereof.

15 Examples of cancer that may be treated according to the present invention include, but are not limited to, leukemias including chronic myelogenous leukemia, acute myelogenous leukemia, acute lymphoblastic leukemia, and T cell and B cell leukemias, lymphomas (Hodgkins and non-Hodgkins), lymphoproliferative disorders, plasmacytomas, histiocytomas, melanomas, adenomas, sarcomas, carcinomas of solid tissues, hypoxic
20 tumours, squamous cell carcinomas, genitourinary cancers such as cervical and bladder cancer, hematopoietic cancers, head and neck cancers, and nervous system cancers.

25 In a preferred embodiment, the present invention provides a method of treating chronic myelogenous leukemia comprising administering an effective amount of TcR $\gamma\delta^+$ T cells prepared according to the method of the invention to an animal in need thereof. In such an embodiment, the LDMNC can be obtained from a patient with chronic myelogenous leukemia (CML). After culturing and expanding for TcR $\gamma\delta^+$ T cells, the expanded cells will not contain cancerous CML cells making them well suited for re-infusion back to
30 the patient.

The invention also includes the use of the TcR $\gamma\delta^+$ T cells obtained by the methods of the invention to modulate an immune response, to treat an infection or to treat cancer as described hereinabove. The invention further

includes the use of the TcR $\gamma\delta^+$ T cells obtained according to the methods of the invention to prepare a medicament or pharmaceutical composition to modulate an immune response, to treat an infection or to treat cancer as described hereinabove.

5 The TcR $\gamma\delta^+$ T cells obtained according to the present invention can also be used in experimental models, for example, to further study and elucidate the function of the cells. Additionally, these cells may be used for studies directed towards the identification of the antigens/epitopes recognized by TcR $\gamma\delta^+$ T cells and for the design and development of vaccines.

10 The obtained TcR $\gamma\delta^+$ T cells, according to the invention may be immediately used in the above therapeutic, experimental or commercial applications or the cells may be cryopreserved for use at a later date.

The following non-limiting examples are illustrative of the present invention:

15 **EXAMPLES**

EXAMPLE 1

Low density mononuclear cells (LDMNC) were isolated from adult peripheral blood by density gradient centrifugation using Ficoll-Hypaque™ (density = 1.077 g/ml). A volume of 100 ml of whole blood was layered in 10
20 15 ml aliquots onto an equal volume of Ficoll-Hypaque™ in 50 ml conical tissue culture tubes which were then centrifuged at 400 x g for 30 minutes at room temperature. The interphase material containing the mononuclear cells was collected and the cells were washed twice in culture medium (AIM-V™ containing 20 units/ml of heparin and 50 µM 2-mercaptoethanol; HCBM-2)
25 by centrifugation at 100 x g for 10 minutes at room temperature. The cells were diluted in HCBM-2 containing 10% autologous plasma and incubated in polystyrene tissue culture flasks overnight at 37°C and 5% CO₂. The next morning, the cells were washed twice by centrifugation and resuspended in phosphate buffered saline (PBS) containing 2% autologous plasma. A sample
30 of the cell suspension was diluted 1:20 with 2% acetic acid and the total number of nucleated cells determined using a hemocytometer.

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TcR $\gamma\delta^+$ T cells were enriched from the LDMNC by negative selection using a cocktail of lineage specific antibodies and immunomagnetic affinity chromatography (StemSep™, Stem Cell Technologies, Vancouver, BC). A total of 8.2×10^7 LDMNC were resuspended in 1.25 ml of PBS containing 2% autologous plasma and a cocktail of lineage specific, monoclonal antibodies was added. The cocktail contained antibodies specific for CD14 (monocytes), CD16 (NK cells), CD19 (B cells), CD33 (myeloid progenitor cells), CD56 (NK cells), glycophorin A (erythroid cells) and TcR $\alpha\beta$ (TcR $\alpha\beta^+$ T cells). These were bispecific antibodies with one antigenic specificity for the lineage specific marker and the other antigenic specificity for dextran. The LDMNC were incubated with the bispecific antibodies on ice for 30 minutes following which iron dextran colloid was added and the incubation was continued for a further 30 minutes. The suspension was then subjected to immunomagnetic chromatography, a procedure which removed those cells which had been cross-linked by the bispecific antibodies to the iron dextran particles. Thus, the cells recovered were an enriched population of TcR $\gamma\delta^+$ T cells that had not been bound by antibody or iron dextran. The yield of enriched TcR $\gamma\delta^+$ T cells obtained was 2.65×10^5 cells.

The enriched TcR $\gamma\delta^+$ T cells were seeded into tissue culture under various conditions (described below) at a density of 1×10^5 cells/ml in HCBM-2 containing 5% autologous plasma (P) and the cultures were incubated at 37°C and 5% CO₂. At various time points, cell expansion was monitored by counting the cells in a small sample of each culture using a hemocytometer. The samples were diluted with an equal volume of 0.4% trypan blue prior to counting, so that the viable cells (unstained) could be distinguished from the non-viable cells (stained blue).

Initially, the cells were cultured under two different conditions (Condition 1 and Condition 6) and then the cells in Condition 1 were sub-cultured under five different conditions (Conditions 1-5). Initially, in Condition 1, a combination of mitogen and cytokines was added to 1.3×10^5 enriched TcR $\gamma\delta^+$ T cells as follows: 1 ug/ml of concanavalin A (ConA), 10

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ng/ml of IL-2 and 10 ng/ml of IL-4. The cells were cultured in this condition for 7 days, after which they had expanded approximately 3.5-fold to a total of 4.5×10^5 viable cells. At this point, fresh culture medium containing 5% autologous plasma was added to the culture to dilute the cells to a density of 1×10^5 cells/ml (total volume = 4.5 ml) and 1 ug/ml of concanavalin A, 10 ng/ml of IL-2 and 10 ng/ml of IL-4 were added. The culture was continued for a further 2 days, after which the cells had expanded to a total of 2.0×10^6 viable cells. At this point, the cells were sub-cultured as follows: the cells were pelleted by centrifugation at $100 \times g$ for 10 minutes at room temperature, washed once with HCBM-2 and resuspended at 1.0×10^5 cells/ml in HCBM-2 containing 2% autologous plasma. The resuspended cells were divided into 5 equal portions of 4.0×10^5 cells each and were cultured under the following conditions:

1. 1 ug/ml ConA + 10 ng/ml IL-2 + 10 ng/ml IL-4 + 2% P
2. 10 ng/ml IL-2 + 10 ng/ml IL-4 + 2% P
3. 10 ng/ml IL-2 + 2% P
4. 10 ng/ml IL-4 + 2% P
5. 2% P

At day 12, the cells in each culture were counted and fresh culture medium containing 2% autologous plasma was added to each to dilute the cells to 1×10^5 cells/ml. Fresh mitogen and cytokines were added to maintain the conditions given above. The cultures were continued for another 4 days (to day 16), at which point the cells in each condition were counted and analyzed by flow cytometry to determine the percentage of TcR $\gamma\delta^+$ T cells.

In a sixth culture condition, the enriched TcR $\gamma\delta^+$ T cells were seeded into culture as described above except in this condition the cells were never stimulated with mitogen and were maintained throughout in the presence of 10 ng/ml of IL-2 and 10 ng/ml of IL-4 in HCBM-2 with autologous plasma.

Summary of Conditions:

Culture Condition \rightarrow Sub-culture Condition

1. Con A + IL-2 + IL-4 + P \rightarrow Con A + IL-2 + IL-4 + P
2. Con A + IL-2 + IL-4 + P \rightarrow IL-2 + IL-4 + P

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3. Con A + IL-2 + IL-4 + P \rightarrow IL-2 + P
4. Con A + IL-2 + IL-4 + P \rightarrow IL-4 + P
5. Con A + IL-2 + IL-4 + P \rightarrow P
6. IL-2 + IL-4 + P

5 The results of this experiment are shown in Figure 1 in which the total number of viable cells in each culture condition is plotted as a function of time. The arrow in the figure indicates the time point at which the cells were sub-cultured. The numerical cell counts are given in the table accompanying the graph, together with the percentage of TcR $\gamma\delta^+$ T cells in the expanded
10 populations at the end of the culture period.

These data demonstrate the expansion of TcR $\gamma\delta^+$ T cells by the method of the present invention, i.e.

Mitogen (e.g. Con A) + IL-2 + IL-4
 \rightarrow IL-2 + IL-4

15 The data also demonstrate that the expansion of TcR $\gamma\delta^+$ T cells is greater when IL-4 is used than when it is not, i.e. the total expansion was higher in condition 2 compared to condition 3.

The data further demonstrate that the mitogen is necessary at the beginning of the culture for significant expansion of TcR $\gamma\delta^+$ T cells to occur,
20 i.e. the total expansion was much higher in condition 2 compared to condition 6.

The data further demonstrate that the sub-culture step (i.e. removal of the mitogen from the culture) results in better expansion of TcR $\gamma\delta^+$ T cells, i.e. the total expansion was higher in condition 2 compared to condition 1.

25 The data also demonstrate that very pure TcR $\gamma\delta^+$ T cells (99%) are obtained by the method of the present invention without the need for further purification of the expanded population of cells.

EXAMPLE 2

30 TcR $\gamma\delta^+$ T cells were enriched from adult peripheral blood LDMNC as described in Example 1. The enriched TcR $\gamma\delta^+$ T cells were seeded into tissue culture and expanded in a manner similar to that described in Example 1,

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condition 2. Specifically, cultures were initiated with (1) 1 ug/ml of concanavalin A and 10 ng/ml of IL-2, or (2) 1 ug/ml of concanavalin A and 10 ng/ml of IL-2 and 10 ng/ml of IL-4, or (3) 1 ug/ml of concanavalin A and 10 ng/ml of IL-2 and 10 ng/ml of IL-7. At day 10, the cells in each condition were sub-cultured, i.e. the mitogen was removed, and the cells were further expanded in (1) IL-2, or (2) IL-2 and IL-4, or (3) IL-2 and IL-7. At day 17, the cultures were terminated, final cell counts were determined and TcR $\gamma\delta^+$ T cell purity was assessed by flow cytometry.

Summary of Conditions:

10 Culture Condition \rightarrow Sub-culture Condition

1. Con A + IL-2 + P \rightarrow IL-2 + P
2. Con A + IL-2 + IL-4 + P \rightarrow IL-2 + IL-4 + P
3. Con A + IL-2 + IL-7 + P \rightarrow IL-2 + IL-7 + P

15 The results of this experiment are shown in Figure 2 in which the total number of viable cells in each culture condition is plotted as a function of time. The arrow in the figure indicates the time point at which the cells were sub-cultured (i.e. the mitogen was removed). The numerical cell counts are given in the table accompanying the graph, together with the percentage of TcR $\gamma\delta^+$ T cells in the expanded populations at the end of the culture period.

20 These data demonstrate the expansion of TcR $\gamma\delta^+$ T cells by the method of the present invention, i.e.

Mitogen (e.g. Con A) + IL-2 + IL-4 or IL-7
 \rightarrow IL-2 + IL-4 or IL-7

25 The data further demonstrate that the expansion of TcR $\gamma\delta^+$ T cells is greater when IL-4 or IL-7 is used than when it is not, i.e. the total expansion was higher in conditions 2 and 3 compared to condition 1.

The data also demonstrate that very pure TcR $\gamma\delta^+$ T cells (>95%) are obtained by the method of the present invention without further purification of the expanded population of cells.

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EXAMPLE 3

TcR $\gamma\delta^+$ T cells were enriched from adult peripheral blood LDMNC as described in Example 1. The enriched TcR $\gamma\delta^+$ T cells were seeded into tissue culture and expanded in a manner similar to that described in Example 1, condition 2, except that the mitogen used was an immobilized monoclonal antibody instead of concanavalin A. The monoclonal anti-CD3 antibody OKT3 was coated onto the wells of a tissue culture plate at a concentration of 10 ug/ml in PBS overnight at 4°C. The wells were emptied and washed five times with PBS prior to use. As a negative control, an isotype-matched immunoglobulin (IgG2a) of irrelevant antigenic specificity was similarly used. Cultures were initiated with (1) immobilized OKT3 and 10 ng/ml of IL-2, (2) immobilized OKT3 and 10 ng/ml of IL-2 and 10 ng/ml of IL-4, or (3) immobilized IgG2a and 10 ng/ml of IL-2 and 10 ng/ml of IL-4. At day 8, the cells in each condition were sub-cultured, i.e. the mitogen was removed. This was accomplished by resuspending the cells, removing the cell suspension from the coated wells, pelleting and washing the cells by centrifugation, and replacing the cells in uncoated tissue culture wells. The cultures were further expanded in (1) IL-2, (2) IL-2 and IL-4, or (3) IL-2 and IL-4. At day 20, the cultures were terminated, final cell counts were determined and TcR $\gamma\delta^+$ T cell purity was assessed by flow cytometry.

Summary of Conditions:

Culture Condition → Sub-culture Condition

1. OKT3 + IL-2 + P → IL-2 + P
2. OKT3 + IL-2 + IL-4 + P → IL-2 + IL-4 + P
- 25 3. IgG2a + IL-2 + IL-4 + P → IL-2 + IL-4 + P

The results of this experiment are shown in Figure 3 in which the total number of viable cells in each culture condition is plotted as a function of time. The arrow in the figure indicates the time point at which the cells were sub-cultured. The numerical cell counts are given in the table accompanying the graph, together with the percentage of TcR $\gamma\delta^+$ T cells in the expanded populations at the end of the culture period.

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These data demonstrate the expansion of TcR $\gamma\delta^+$ T cells by the method of the present invention, i.e.

Mitogen (e.g. OKT3) + IL-2 + IL-4

→ IL-2 + IL-4

5 The data demonstrate that the expansion of TcR $\gamma\delta^+$ T cells is greater when IL-4 is used than when it is not, i.e. the total expansion was higher in condition 2 compared to condition 1.

 The data demonstrate that the mitogen can be a monoclonal antibody, and that the specificity of the monoclonal antibody is important since the total
10 expansion was much higher in condition 2 compared to condition 3.

 The data also demonstrate that very pure TcR $\gamma\delta^+$ T cells (94%) are obtained by the method of the present invention without further purification of the expanded population of cells. Moreover, the purity of the TcR $\gamma\delta^+$ T cells is higher when IL-4 is used (94%) than when it is not (73%).

15 **EXAMPLE 4**

 TcR $\gamma\delta^+$ T cells were enriched from adult peripheral blood LDMNC as described in Example 1. The enriched TcR $\gamma\delta^+$ T cells were seeded into tissue culture and expanded in a manner similar to that described in Example 3. Cultures were initiated with (1) immobilized OKT3 and 10 ng/ml of IL-2, (2)
20 immobilized OKT3 and 10 ng/ml of IL-2 and 10 ng/ml of IL-4, or (3) immobilized OKT3 and 10 ng/ml of IL-2 and 10 ng/ml of IL-7. At day 6, the cells in each condition were sub-cultured, i.e. the mitogen was removed. The cultures were further expanded in (1) IL-2, (2) IL-2 and IL-4, or (3) IL-2 and IL-7. At day 21, the cultures were terminated, and final cell counts were
25 determined.

Summary of Conditions:

Culture Condition → Sub-culture Condition

1. OKT3 + IL-2 + P → IL-2 + P
2. OKT3 + IL-2 + IL-4 + P → IL-2 + IL-4 + P
- 30 3. OKT3 + IL-2 + IL-7 + P → IL-2 + IL-7 + P

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The results of this experiment are shown in Figure 4 in which the total number of viable cells in each culture condition is plotted as a function of time. The arrow indicates the time point at which the cells were sub-cultured. The numerical cell counts are given in the table accompanying the graph,
 5 together with the percentage of TcR $\gamma\delta^+$ T cells in the expanded populations at the end of the culture period.

These data demonstrate the expansion of TcR $\gamma\delta^+$ T cells by the method of the present invention, i.e.

10 Mitogen (e.g. OKT3) + IL-2 + IL-4 or IL-7
 \rightarrow IL-2 + IL-4 or IL-7

The data further demonstrate that the expansion of TcR $\gamma\delta^+$ T cells is greater when IL-4 or IL-7 is used than when it is not, i.e. the total expansion was higher in conditions 2 and 3 compared to condition 1.

EXAMPLE 5

15 TcR $\gamma\delta^+$ T cells were enriched from adult peripheral blood LDMNC as described in Example 1. The enriched TcR $\gamma\delta^+$ T cells were seeded into tissue culture and expanded in a manner similar to that described in Example 2. Specifically, cultures were initiated with 1 ug/ml of concanavalin A and a combination of cytokines (see below), each of which was used at 10 ug/ml. At
 20 day 8, the cells in each condition were sub-cultured, i.e. the mitogen was removed, and the cells were further expanded using the respective combination of cytokines alone.

Summary of Conditions:

	Culture Condition \rightarrow Sub-culture Condition
25	1. Con A + IL-2 + P \rightarrow IL-2 + P
	2. Con A + IL-2 + IL-4 + P \rightarrow IL-2 + IL-4 + P
	3. Con A + IL-2 + IL-4 + IL-7 + P \rightarrow IL-2 + IL-4 + IL-7 + P
	4. Con A + IL-2 + IL-4 + IL-15 + P \rightarrow IL-2 + IL-4 + IL-15 + P
	5. Con A + IL-2 + IL-7 + IL-15 + P \rightarrow IL-2 + IL-7 + IL-15 + P
30	6. Con A + IL-2 + IL-4 + IL-7 + IL-15 + P \rightarrow IL-2 + IL-4 + IL-7 + IL-15 + P

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The results of this experiment are shown in Figure 5 in which the total number of viable cells in each culture condition is plotted as a function of time. The arrow in the figure indicates the time point at which the cells were sub-cultured (i.e. the mitogen was removed). The numerical cell counts are
5 given in the table accompanying the graph.

These data demonstrate that the addition of a third growth factor enhances the expansion of the TcR $\gamma\delta^+$ T cells as compared to the expansion obtained using one or two growth factors, i.e. the expansion was higher in conditions 3, 4 and 5, compared to conditions 1 and 2.

10 These data further demonstrate that the addition of a fourth growth factor enhances the expansion of the TcR $\gamma\delta^+$ T cells as compared to the expansion obtained using one or two or three growth factors, i.e. the expansion was higher in condition 6 compared to conditions 1 - 5.

While the present invention has been described with reference to what
15 are presently considered to be the preferred examples, it is to be understood that the invention is not limited to the disclosed examples. To the contrary, the invention is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

All publications, patents and patent applications are herein
20 incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

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FULL CITATIONS FOR REFERENCES REFERRED TO IN THE SPECIFICATION

- Bensussan, A., Lagabrielle, J.F., Castaigne, S., Boisson, N., Miclea, J.M.,
5 Benbunan, M., Degos, L. Human CD3 gamma delta + activated lymphocytes exhibit killer activity *in vitro* against autologous leukemia cells. *Nouv Rev Fr Hematol* 31:129, 1989.
- Boismenu, R., Havran, W.L. An innate view of $\gamma\delta$ T cells. *Curr Op Immunol*
10 9:57, 1997.
- Bukowski, J.F., Morita, C.T., Brenner, M.B. Recognition and destruction of virus-infected cells by human $\gamma\delta$ CTL. *J. Immunol* 153:5133, 1994.
- 15 Choudhary, A., Davodeau, F., Moreau, A., Peyrat, M.A., Bonneville, M., Jotereau, F. Selective lysis of autologous tumor cells by recurrent $\gamma\delta$ tumor-infiltrating lymphocytes from renal carcinoma. *J Immunol* 154:3932, 1995.
- Constant, P., Davodeau, F., Peyrat, M., Poquet, Y., Puzo, G., Bonneville, M.
20 and Fournie, J. Stimulation of human $\gamma\delta$ T cells by nonpeptidic mycobacterial ligands. *Science* 264:267, 1994.
- Elloso, M.M., Van der Heyde, H.C., Troutt, A., Manning, D.D. and Weidanz, W.P. Human gamma delta T cell subset-proliferative response to malarial
25 antigen *in vitro* depends on CD4⁺ T cells or cytokines that signal through components of the IL-2R. *J. Immunol.* 157:2096, 1996.
- Garcia, V.E., Jullien, D., Song, M., Uyemura, K., Shuai, K, Morita, C.T. and Modlin, R.L. IL-15 enhances the response of human gamma delta T cells to
30 non-peptide microbial antigens. *J. Immunol.* 160:4322, 1998.
- Jahn, B., Bergmann, L., Weidmann, E., Brieger, J., Fenchel, K., Schwulera, U., Hoelzer, D., Mitrou, P.S. Bone marrow-derived T-cell clones obtained from

- 23 -

untreated acute myelocytic leukemia exhibit blast directed autologous cytotoxicity. Leuk Res 19:73, 1995.

- 5 Kaur, I., Voss, S.D., Gupta, R.S., Schell, K., Fisch, P. and Sondel, P.M. Human peripheral $\gamma\delta$ T cells recognize hsp60 molecules on Daudi Burkitt's lymphoma cells. J. Immunol. 150:2046, 1993.

- 10 Kitayama, J., Atomi, Y., Nagawa, H., Kuroda, A., Mutoh, T., Minami, M., Juji, T. Functional analysis of TCR $\gamma\delta^+$ T cells in tumour-infiltrating lymphocytes (TIL) of human pancreatic cancer. Clin Exp Immunol 93:442, 1993.

- 15 Lamb, L.S., Henslee-Downey, P.J., Parrish, R.S., Godder, K., Thompson, J., Lee, C., Gee, A.P. Increased frequency of TCR gamma delta + T cells in disease-free survivors following T cell-depleted, partially mismatched, related donor bone marrow transplantation for leukemia. J Hematother 5:503, 1996.

Lang, F., Peyrat, M.A., Constant, P., Davodeau, F., David-Ameline, J., Poquet, Y., Vie, H., Fournie, J.J., Bonneville, M. J Immunol 154:5986, 1995.

- 20 Orsini, D.L.M., Res, P.C.M., Van Laar, J.M., Muller, L.M., Soprano, A.E.L., Kooy, Y.M.C., Tak, P.P. and Koning, F. A subset of V δ 1⁺ T cells proliferates in response to Epstein-Barr virus-transformed B cell lines *in vitro*. Scand. J. Immunol. 38:335, 1993.

- 25 Penninger, J., Wen, T., Timms, E., Potter, J., Wallace, V.A., Matsuyama, T., Ferrick, D., Sydora, B., Kronenberg, M. Mak, T.W. Spontaneous resistance to acute T-cell leukaemias in TCR δ 1.1 γ 4C γ 4 transgenic mice. Nature 375:241, 1995.

- 30 Salerno, A. and Dieli, F. Role of $\gamma\delta$ T lymphocytes in immune response in humans and mice. Crit. Rev. Immunol. 18:327, 1998.

- 24 -

Suzuki, Y., Fujimiya, Y., Ohno, T., Katakura, R. and Yoshimoto, T. Enhancing effect of tumor necrosis factor (TNF)- α , but not IFN- γ , on the tumor-specific cytotoxicity of $\gamma\delta$ T cells from glioblastoma patients. *Cancer Lett.* 140:161, 1999.

- 5 Wallace, M., Malkovsky, M., Carding, S.R. Gamma/delta T lymphocytes in viral infections. *J. Leuk Biol* 58:277, 1995.

Yamaguchi, T., Fujimiya, Y., Suzuki, Y., Katakura, R. and Ebina, T. A simple method for the propagation and purification of $\gamma\delta$ T cells from the peripheral
10 blood of glioblastoma patients using solid-phase anti-CD3 antibody and soluble IL-2. *J. Immunol. Meth.* 205:19, 1997.

Yamaguchi, T., Suzuki, Y., Katakura, R., Ebina, T., Yokoyama, J. and Fujimiya, Y. Interleukin-15 effectively potentiates the in vitro tumor-specific activity and
15 proliferation of peripheral blood $\gamma\delta$ T cells isolated from glioblastoma patients. *Cancer Immunol. Immunother.* 47:97, 1998.

Yu, S., He, W., Chen, J., Zhang, F. and Ba, D. Expansion and immunological study of human tumor infiltrating gamma/delta T lymphocytes *in vitro*. *Int.*
20 *Arch. Allergy Immunol.* 119:31, 1999.

Zocchi, M.R., Ferrarini, M., Rugarli, C. Selective lysis of the autologous tumor by δ TCS1 + $\gamma\delta$ + tumor-infiltrating lymphocytes from human lung carcinomas. *Eur J Immunol* 20:2685, 1990.

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WE CLAIM:

1. A method for expanding TcR $\gamma\delta^+$ T cells in a starting sample comprising:
 - 5 (1) culturing cells in the starting sample in a first culture medium comprising a T cell mitogen, a growth factor having interleukin-2-like activity and a growth factor having interleukin-7-like activity; and
 - (2) culturing the cells obtained in step (1) in a second culture medium comprising a growth factor having interleukin-2-like activity and
10 a growth factor having interleukin-7-like activity to expand TcR $\gamma\delta^+$ T cells.
2. A method according to claim 1 wherein the growth factor having interleukin-2-like activity is interleukin-2.
15
3. A method according to claim 1 wherein the growth factor having interleukin-7-like activity is interleukin-7.
4. A method according to any one of claims 1 to 3 wherein prior to step
20 (1) the cells in the starting sample are enriched for T cells.
5. A method according to any one of claims 1 to 4 wherein prior to step (1) the cells in the starting sample are enriched for TcR $\gamma\delta^+$ T cells.
- 25 6. A method according to any one of claims 1 to 5 wherein prior to step (1) the cells in the starting sample are depleted of CD14 $^+$, CD16 $^+$, CD19 $^+$, CD33 $^+$, CD56 $^+$ and glycophorin A $^+$ cells.
7. A method according to any one of claims 1 to 6 wherein prior to step
30 (1) the cells in the starting sample are depleted of TcR $\alpha\beta^+$ T cells.
8. A method according to any one of claims 1 to 7 wherein prior to step (1) the cells in the starting sample are depleted of non-TcR $\gamma\delta^+$ T cells.

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9. A method according to any one of claims 1 to 8 wherein the starting sample is blood or tissue or fractions thereof.
- 5 10. A method according to claim 9 wherein the starting sample is selected from peripheral blood, umbilical cord blood, bone marrow, lymphoid tissue, epithelia, thymus, liver, spleen, cancerous tissue, infected tissue, lymph node tissue or fractions thereof.
- 10 11. A method according to claim 9 or 10 wherein the starting sample is human peripheral blood or a fraction thereof.
12. A method according to any one of claims 1 to 11 wherein the starting sample is low density mononuclear cells.
- 15 13. A method according to any one of claims 1 to 12 wherein in the first culture medium the T cell mitogen is present in an amount from about 0.01 to about 100 µg/ml and wherein in the first and second culture media the growth factor having interleukin-2-like activity is present in an amount from about 0.1 to about 1000 ng/ml and the growth factor having interleukin-7-like activity is present in an amount from about 0.1 to about 1000 ng/ml.
- 20 14. A method according to any one of claims 1 to 12 wherein in the first culture medium the T cell mitogen is present in an amount from about 0.1 to about 50 µg/ml and wherein in the first and second culture media the growth factor having interleukin-2-like activity is present in an amount from about 1 to about 100 ng/ml and the growth factor having interleukin-7-like activity is present in an amount from about 1 to about 100 ng/ml.
- 25 15. A method according to any one of claims 1 to 12 wherein in the first culture medium the T cell mitogen is present in an amount from about 0.5 to about 10 µg/ml and wherein in the first and second culture media the growth factor having interleukin-2-like activity is present in an amount from about 2

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to about 50 ng/ml and the growth factor having interleukin-7-like activity is present in an amount from about 2 to about 50 ng/ml.

16. A method according to any one of claims 1 to 15 wherein the first
5 culture medium comprises 1 µg/mL of a T cell mitogen and wherein in the first and second culture media 10 ng/mL of a growth factor having interleukin-2-like activity and 10 ng/mL of a growth factor having interleukin-7-like activity.
- 10 17. A method according to any one of claims 1 to 16 wherein said first and second culture media further comprises a third growth factor.
18. A method according to claim 17 wherein said third growth factor is IL-4 or IL-15 or a mimetic or functional equivalent thereof.
- 15 19. A method according to any one of claims 1 to 16 wherein said first and second culture media further comprises a third and a fourth growth factor.
20. A method according to claim 19 wherein said third and fourth growth
20 factors are IL-4 and IL-15, or a mimetic or functional equivalent thereof.
21. A method according to any one of claims 1 to 20 wherein the T cell mitogen is a plant lectin.
- 25 22. A method according to claim 21 wherein the plant lectin is concanavalin A.
23. A method according to any one of claims 1 to 20 wherein the T cell mitogen is an antibody or a fragment thereof.
- 30 24. A method according to claim 23 wherein the antibody binds to CD3 or a fragment thereof.

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25. A method according to any one of claims 1 to 24 wherein the first and second culture media further contains serum or plasma.
26. A method according to claim 25 wherein the serum or plasma is
5 present in an amount from about 1 to about 25% by volume.
27. A method according to claim 25 wherein the serum or plasma is present in an amount from about 2 to about 20% by volume.
- 10 28. A method according to claim 25 wherein the serum or plasma is present in an amount from about 2.5 to about 10% by volume.
29. A method according to claim 25 wherein the serum or plasma is present in an amount of about 5% by volume.
- 15 30. A cell preparation enriched in TcR $\gamma\delta^+$ T cells prepared according to the method of any one of claims 1 to 29.
31. A cell preparation enriched in TcR $\gamma\delta^+$ T cells wherein greater than 70%
20 of the total cells are TcR $\gamma\delta^+$ T cells.
32. A cell preparation according to claims 30 or 31 wherein greater than 80% of the total cells are TcR $\gamma\delta^+$ T cells.
- 25 33. A cell preparation according to claims 30, 31 or 32 wherein greater than 90% of the total cells are TcR $\gamma\delta^+$ T cells.
34. A cell preparation according to any one of claims 30 to 33 that is substantially free of a T cell mitogen.
- 30 35. A use of a cell preparation according to any one of claims 30 to 34 to prepare a medicament to modulate an immune response.

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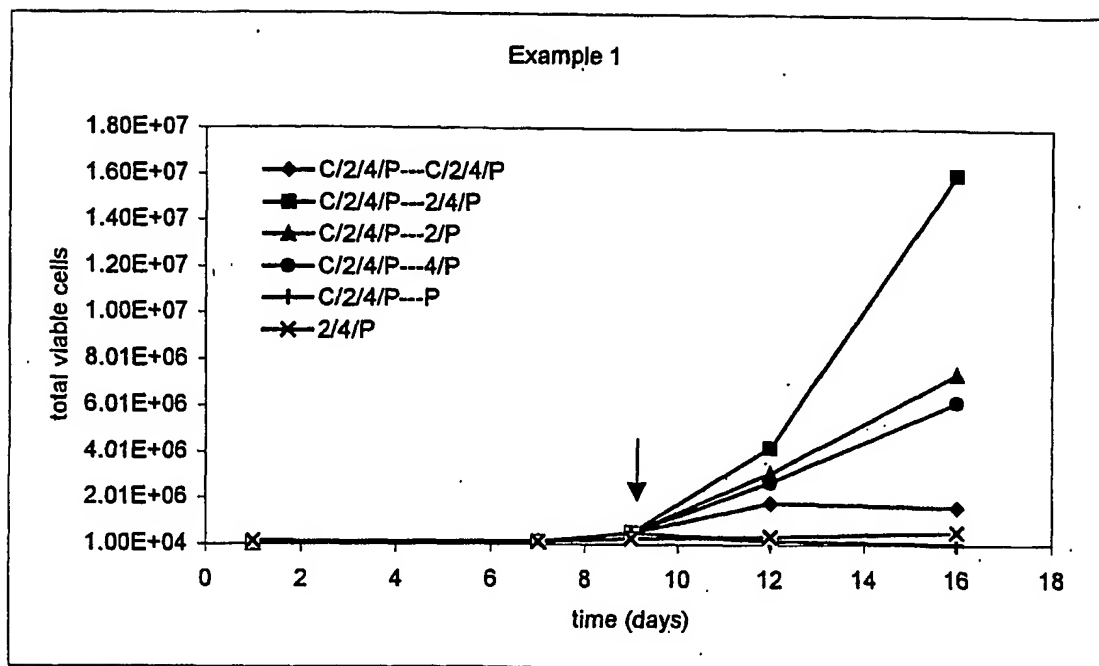
36. A use of a cell preparation according to any one of claims 30 to 34 to prepare a medicament to treat an infection.
37. A use of a cell preparation according to any one of claims 30 to 34 to
5 prepare a medicament to treat cancer.
38. A use of a cell preparation according to any one of claims 30 to 34 to prepare a medicament to treat chronic myelogenous leukemia.
- 10 39. A use of a cell preparation according to any one of claims 30 to 34 to prepare a vaccine.
40. A use of a cell preparation according to any one of claims 30 to 34 to study antigen recognition, activation, signal transduction or function of
15 TcR $\gamma\delta^+$ T cells.
41. A method of modulating an immune response comprising administering an effective amount of TcR $\gamma\delta^+$ T cells obtained according to the method of any one of claims 1 to 29 or obtained from a cell preparation
20 according to any one of claims 30 to 34 to an animal in need thereof.
42. A method for treating an infection comprising administering an effective amount of TcR $\gamma\delta^+$ T cells obtained according to the method of any one of claims 1 to 29 or obtained from a cell preparation according to any one
25 of claims 30 to 34 to an animal in need thereof.
43. A method for treating cancer comprising administering an effective amount of TcR $\gamma\delta^+$ T cells obtained according to the method of any one of claims 1 to 29 or obtained from a cell preparation according to any one of
30 claims 30 to 34 to an animal in need thereof.
44. A method for treating chronic myelogenous leukemia comprising administering an effective amount of TcR $\gamma\delta^+$ T cells obtained according to the

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method of any one of claims 1 to 29 or obtained from a cell preparation according to any one of claims 30 to 34 to an animal in need thereof.

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FIGURE 1



EXPT. Condition	Total Viable Cells					Percent TcR γ δ +
	1 day	7 days	9 days	12 days	16 days	
C/2/4/P---C/2/4/P	2.60E+04	1.10E+05	5.00E+05	1.80E+06	1.60E+06	97
C/2/4/P---2/4/P	2.60E+04	1.10E+05	5.00E+05	4.20E+06	1.60E+07	99
C/2/4/P---2/P	2.60E+04	1.10E+05	5.00E+05	3.10E+06	7.40E+06	99
C/2/4/P---4/P	2.60E+04	1.10E+05	5.00E+05	2.70E+06	6.20E+06	98
C/2/4/P---P	2.60E+04	1.10E+05	5.00E+05	1.60E+05	1.90E+04	nd
2/4/P	1.30E+05	1.10E+05	2.50E+05	3.40E+05	5.30E+05	56

C = Concanavalin A

2 = Interleukin-2

4 = Interleukin-4

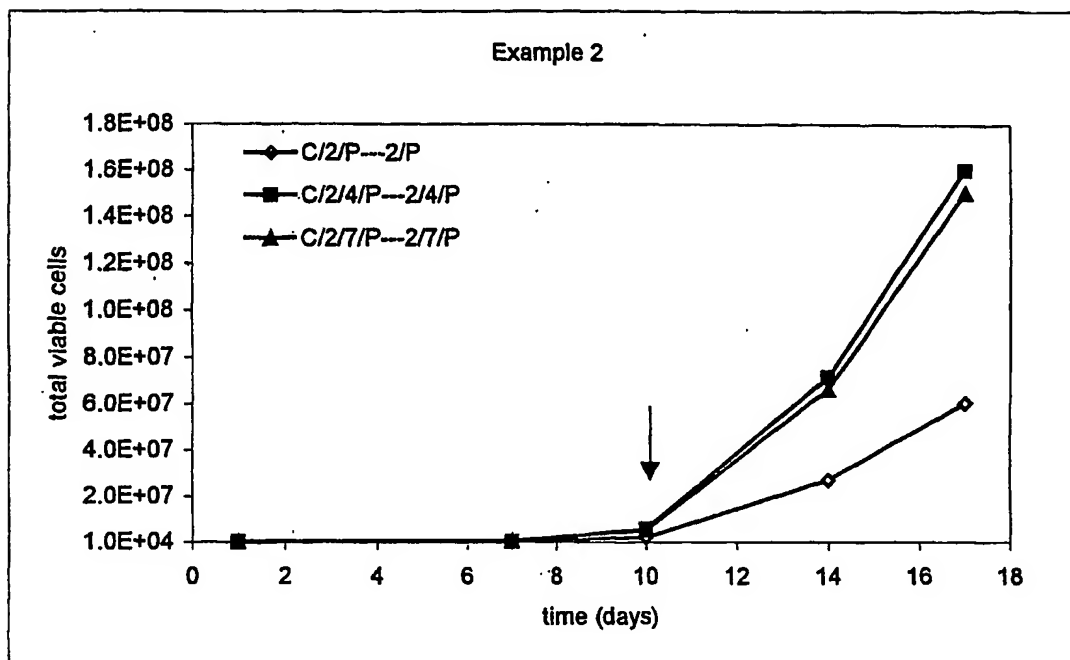
P = Autologous plasma

--- = sub-culture

nd = not done

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FIGURE 2

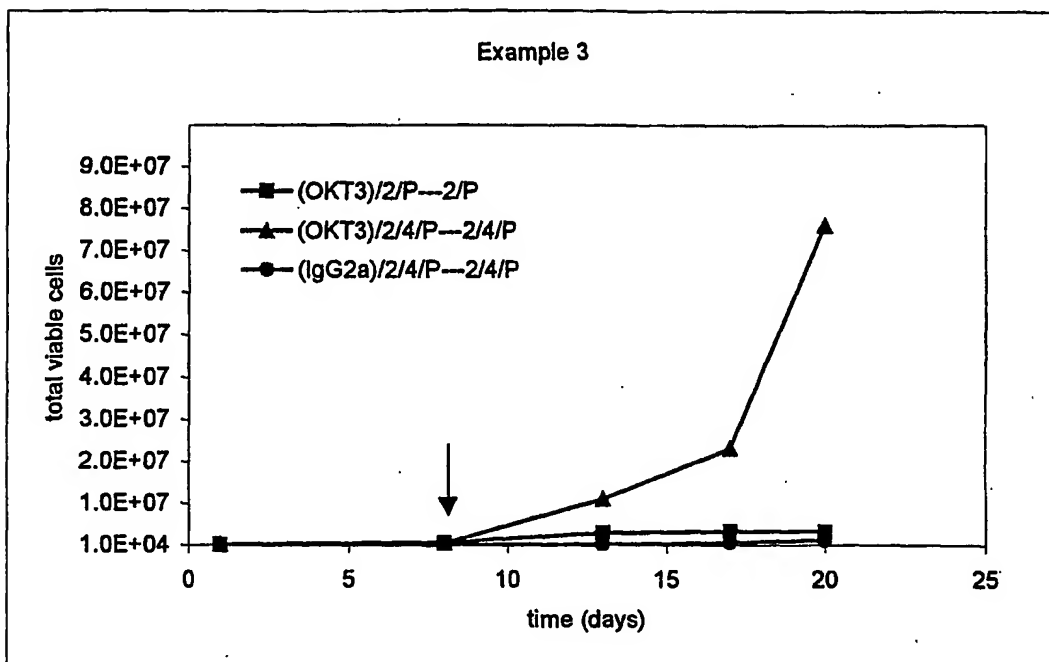


EXPT. Condition	Total Viable Cells					Percent TcR γ δ +
	1 day	7 days	10 days	14 days	17 days	
C/2/P---2/P	4.10E+04	2.40E+05	1.90E+06	2.70E+07	6.00E+07	91
C/2/4/P---2/4/P	4.10E+04	3.10E+05	5.60E+06	7.10E+07	1.60E+08	95
C/2/7/P---2/7/P	4.10E+04	4.30E+05	5.20E+06	6.60E+07	1.50E+08	95

C = Concanavalin A
 2 = Interleukin-2
 4 = Interleukin-4
 7 = Interleukin-7
 P = Autologous plasma
 --- = sub-culture

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FIGURE 3



EXPT. Condition	Total Viable Cells					Percent TcRyδ+
	1 day	8 days	13 days	17 days	20 days	
O/2/P---2/P	8.00E+04	5.70E+05	2.90E+06	3.30E+06	3.10E+06	73
O/2/4/P---2/4/P	8.00E+04	4.60E+05	1.10E+07	2.30E+07	7.60E+07	94
G/2/4/P---2/4/P	8.00E+04	7.80E+04	1.50E+05	5.00E+05	1.10E+06	88

O = OKT3

G = IgG2a

2 = Interleukin-2

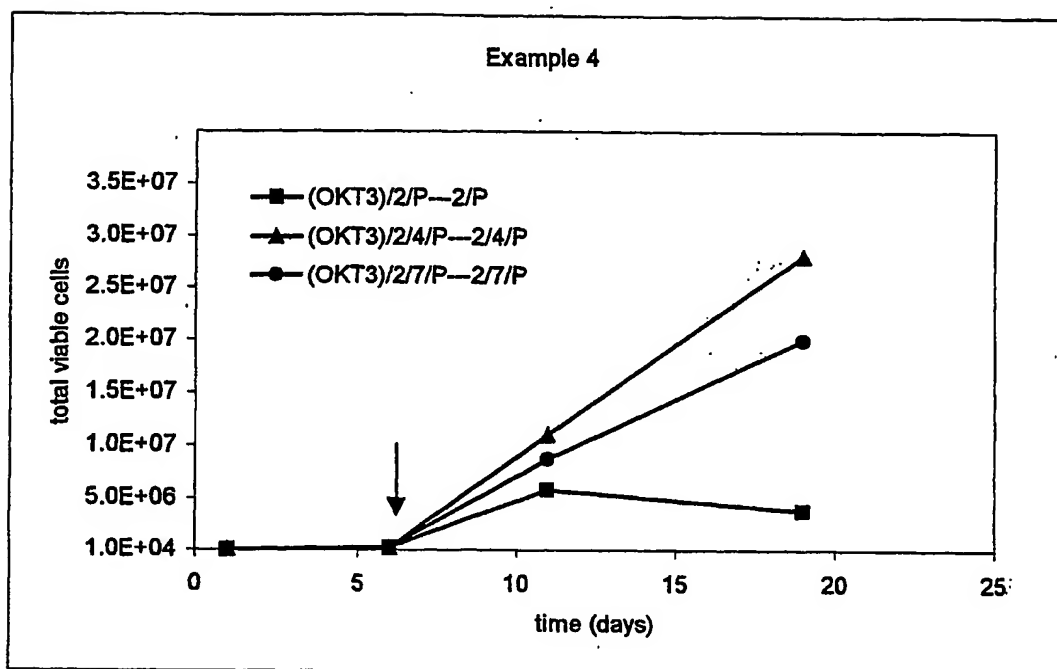
4 = Interleukin-4

P = Autologous plasma

--- = sub-culture

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FIGURE 4

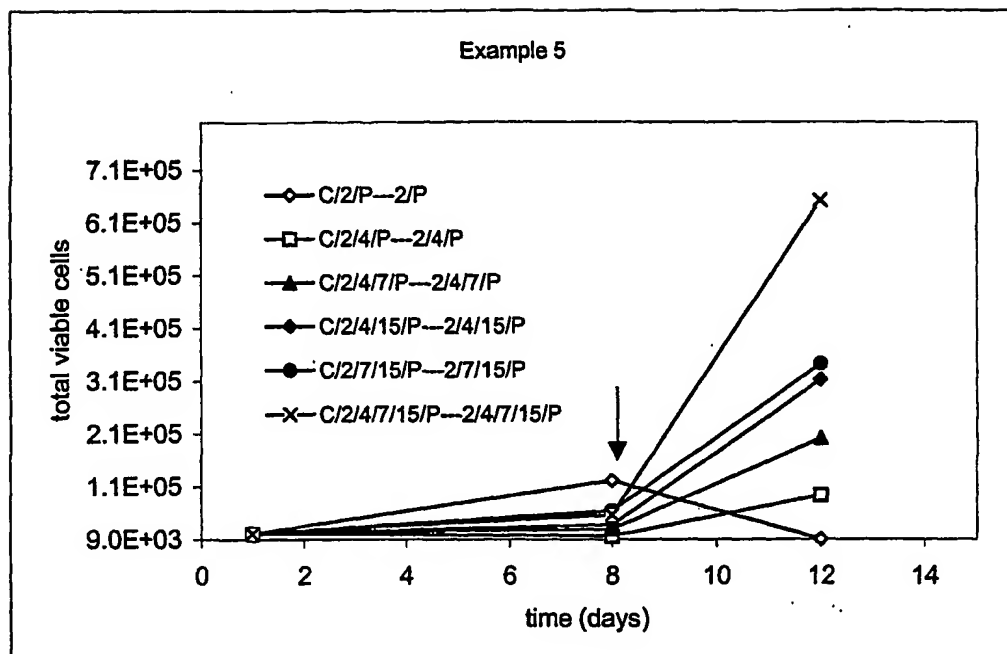


EXPT. Condition	Total Viable Cells				
	1 day	6 days	11 days	19 days	21 days
O/2/P--2/P	3.00E+04	2.50E+05	5.80E+06	3.80E+06	3.40E+06
O/2/4/P--2/4/P	3.00E+04	1.80E+05	1.10E+07	2.80E+07	6.80E+06
O/2/7/P--2/7/P	3.00E+04	2.50E+05	8.70E+06	2.00E+07	9.60E+06

O = OKT3
 2 = Interleukin-2
 4 = Interleukin-4
 7 = Interleukin-7
 P = Autologous plasma
 -- = sub-culture

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FIGURE 5



EXPT. Condition	Total Viable Cells		
	1 day	8 days	12 days
C/2/P---2/P	2.00E+04	8.00E+03	9.00E+03
C/2/4/P---2/4/P	2.00E+04	1.50E+04	9.20E+04
C/2/4/7/P---2/4/7/P	2.00E+04	2.80E+04	2.00E+05
C/2/4/15/P---2/4/15/P	2.00E+04	3.60E+04	3.10E+05
C/2/7/15/P---2/7/15/P	2.00E+04	6.30E+04	3.40E+05
C/2/4/7/15/P---2/4/7/15/P	2.00E+04	5.50E+04	6.50E+05

C = Concanavalin A
 2 = Interleukin-2
 4 = Interleukin-4
 7 = Interleukin-7
 15 = Interleukin-15
 P = Autologous plasma
 --- = sub-culture